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Published in:
Beverages

DOI:
[10.3390/beverages4020040](https://doi.org/10.3390/beverages4020040)

Publication date:
2018


Document version
Publisher's PDF, also known as Version of record

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Citation for published version (APA):
Owusu-Kwarteng, J., Wuni, A., Akabanda, F., & Jespersen, L. (2018). Prevalence and characteristics of *Listeria monocytogenes* isolates in raw milk, heated milk and Nunu, a spontaneously fermented milk beverage in Ghana. *Beverages*, 4(2), [40]. <https://doi.org/10.3390/beverages4020040>

Article

Prevalence and Characteristics of *Listeria monocytogenes* Isolates in Raw Milk, Heated Milk and Nunu, a Spontaneously Fermented Milk Beverage, in Ghana

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Received: 20 April 2018; Accepted: 21 May 2018; Published: 23 May 2018



Abstract: *Listeria monocytogenes* is a gram-positive food-borne pathogen that causes listeriosis in humans. Currently, there is little information on the prevalence of *Listeria monocytogenes* in raw milk and traditional yoghurt-like milk beverage, nunu, in Ghana. The purpose of this study was to investigate the prevalence of *L. monocytogenes* isolates in raw cow milk, boiled milk and nunu in Ghana, and to characterize these *L. monocytogenes* isolates according to their serogroups, virulence potentials and antibiotic susceptibility profiles. A total of 254 samples comprising 114 raw cow milk, 56 boiled milk and 84 nunu were collected from dairy farms and market vendors for detection of *L. monocytogenes*. The overall prevalence of *L. monocytogenes* in raw milk, boiled milk and nunu was 5.5% (14/254). *Listeria monocytogenes* was prevalent in raw cow milk (8.8%; 10/114) and nunu (13.1%; 11/84), while no *Listeria* spp. was not detected in boiled milk. A total of 62 *L. monocytogenes* isolates were analysed to belong to molecular serogroups 1/2a-3a (32/62, 51.6%), 1/2b-3b-7 (14/62, 22.6%), 4b-4d-4e (9/62, 14.5%) and 1/2c-3c (7/62, 11.3%). All 62 *L. monocytogenes* isolates harbored the virulence-associated genes *inlA*, *inlB*, *inlC*, *inlJ*, *plcA*, *actA*, *hlyA*, *iap* and *prfA*. All *Listeria monocytogenes* in the present study were generally susceptible to the tested antibiotics, except neomycin and tetracycline, for which phenotypic resistance was observed among isolates.

Keywords: dairy products; food safety; virulence genes; antimicrobial resistance; *listeria*

1. Introduction

Listeria monocytogenes is a gram positive, facultative intracellular food-borne pathogen that can cause listeriosis in humans, especially in people of compromised immunity, including the elderly, pregnant women and newborns [1,2]. The bacterium is widespread in nature and can survive and grow under low temperatures and pH, high concentrations of salt or bile, oxidative stress, carbon starvation, and other adverse conditions making it a potential hazard in foods [3]. *L. monocytogenes* has been isolated from different raw and ready-to-eat (RTE) foods and in raw milk and dairy products in different countries [4–6]. Several cases of listeriosis in humans are reported, sometimes with a high case-fatality rates [7].

Currently, thirteen (13) different serotypes of *L. monocytogenes* strains have been identified but serotypes 1/2a, 1/2b, 1/2c and 4b are responsible for about 95% of human listeriosis [8,9]. To evaluate

the potential implications of isolated *L. monocytogenes* for food safety and public health, it is necessary to differentiate between virulent and non-virulent strains [10]. Many supposed virulence markers such as internalins (*inlA*, *inlB*, *inlC*, *inlJ*), listeriolysin O (*hlyA*), actin (*actA*), phosphatidylinositol-phospholipase C (PI-PLC, *plcA*), Iap (invasion associated protein, *iap*) and virulence regulator (*prfA*) have been implicated in the pathogenicity of *L. monocytogenes* [11–13].

Generally, *L. monocytogenes* isolates are susceptible to many antibiotics, especially ampicillin/penicillin which are the primary antibiotics for treating listeriosis [14,15]. However, resistance to single or multiple antibiotics have recently been reported for *L. monocytogenes* strains isolated from various food and environmental sources [6,16–18]. It is, therefore, necessary to intensify research aimed at increasing the available data on the prevalence and antibiotic susceptibility of *L. monocytogenes* isolates from various food and environmental sources around the world.

In Ghana, cow milk may be consumed in its raw form, heated or processed by spontaneous fermentation into a yoghurt-like beverage known as nunu [19–21]. Poor control measures in the Ghanaian dairy chain potentially exposes raw cow milk and its products to possible contamination by pathogenic microorganisms such as *L. monocytogenes*, which has implications for the safety of consumers of raw milk and traditional dairy products in Ghana. Appiah et al. [20] reported on the quantitative probabilistic assessment of *L. monocytogenes* exposure among consumers of milk in Ghana. In general, however, there is limited data that can be used for the qualitative and/or quantitative assessment of the risk of *L. monocytogenes* infection associated with the consumption of raw milk and traditional dairy products in Ghana. The problem of limited data is compounded by the lack of a proper surveillance systems for food-borne diseases to provide reliable information on the burden of food-borne illnesses, particularly those involving *L. monocytogenes* associated with milk and milk products in Ghana. Consequently, a number of illnesses or sporadic outbreaks associated with the consumption of *Listeria* contaminated raw milk and milk products may go unreported. Therefore, the objectives of this study were to determine the prevalence of *L. monocytogenes* in raw milk, heated milk and nunu in Ghana and to characterize the isolated *L. monocytogenes* according to their molecular serogroups, antibiotic susceptibility profiles and virulence potential.

2. Materials and Methods

2.1. Samples

A total of 326 samples comprising 114 raw cow milk, 56 boiled milk and 84 nunu (spontaneously fermented yoghurt-like milk beverage) were purchased from dairy farms and open markets in the Northern Region of Ghana between September 2015 and July 2016. Raw milk samples were collected within 10 min. of milking at the dairy farm while boiled milk and nunu samples were collected at point of sale in the market. Prior to sampling, all samples were stored at ambient temperatures (28 ± 2 °C). All samples were collected aseptically and immediately transported in a cool box stored at 4.0 ± 0.5 °C to the laboratory (Savana Agricultural Research Institute, Tamale-Ghana) for analysis. Samples were kept at 4 °C and analyzed within 24 h of collection. The pH of samples was determined upon delivery to the laboratory using a pH meter (Crison basic 20, Barcelona, Spain) calibrated with standard buffers.

2.2. Isolation and Characterization of *L. monocytogenes*

Isolation and identification of *L. monocytogenes* in this study was carried out as described by Becker et al. [22]. Briefly, a 25 g or 25 mL of each sample was aseptically homogenized in 225 mL of *Listeria* enrichment broth base (CM 0862, Oxoid Ltd., Basingstoke, UK) with selective enrichment supplement (SR 0141, Oxoid Ltd., Basingstoke, UK) in Stomacher bags (Seward Ltd., Worthing, UK) for 30 s using a Stomacher (BagMixer, Buch & Holm A/S, Interscience, 78860 St Nom, France), followed by incubation at 30 °C for 24 h. Second enrichment was done by adding 0.1 mL of the overnight culture to 10 mL of *Listeria* enrichment broth base (Oxoid, Basingstoke, UK) with selective enrichment supplement (Oxoid, Basingstoke, UK) and incubated at 37 °C for 48 h. Subsequently, 0.1 mL of the

enriched broth was surface plated on *Listeria* selective oxford agar (CM0856, Oxoid, Basingstoke, UK) plates supplemented with *Listeria* selective supplement (SR0140, Oxoid, Basingstoke, UK). Inoculated plates were incubated at 37 °C for 24–48 h. Presumptive *Listeria* spp. were identified on oxford agar plates after 24 h incubation as colonies with approximately 1 mm diameter, grey to black colonies surrounded by a black halo. Following 48-h incubation period, typical *Listeria* species colonies were approximately 2–3 mm diameter, black with a black halo and sunken center.

For biochemical identification of *L. monocytogenes*, up to five (5) presumptive colonies from each positive plate were streaked on tryptic soy agar (Oxoid, Basingstoke, UK) supplemented with 0.6% yeast extract (Oxoid, Basingstoke, UK) (TSA-YE) and incubated at 35 °C for 24 h. The colonies from TSA-YE plates were confirmed by biochemical tests including Gram staining, catalase, oxidase, triple sugar iron (TSI), sulphide-idole-motility (SIM), methyl-red Voges-Proskauer (MR-VP) reaction, nitrate reduction, and production of acid from rhamnose, xylose, mannitol and α -methyl-D-mannopyranoside. *Listeria monocytogenes* ATCC 19115 was used as a reference strain for biochemical tests and PCR analysis.

2.3. PCR-Based Identification of *L. monocytogenes*

Following biochemical characterization of *Listeria* isolates, identities of *L. monocytogenes* were further confirmed polymerase chain reaction (PCR). Crude DNA was extracted from *Listeria* isolated in this study and *L. monocytogenes* ATCC 19115, used as positive control, by direct boiling of a suspension of the cell lysates [23]. PCR was performed to detect the presence of *Listeria* spp. (LI1/U1) using the specific primer sequences (5'-CTCCATAAAGGTGACCCT-3') and (5'-CAGCMGCCGCGGTAATC-3') and *L. monocytogenes* (LM1/LM2) primer sequences (5'-CCTAAGACGCCAATCGAA-3') and (5'-AAGCGCTTGCAACTGCTC-3') [24]. The primers LI1/U1 and LM1/LM2 were used to amplify the highly conserved 16S rRNA gene (938 bp) of all *Listeria* spp. and listeriolysin O gene (702 bp) to detect *L. monocytogenes*, respectively. The PCR reaction was performed in an automatic thermal cycler (Biotron, Göttingen, Germany) under the following optimized cycling conditions: initial denaturation step of 4 min at 95 °C; 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 45 s, extension at 72 °C for 2 min; and a final elongation at 72 °C for 8 min. The PCR products were separated by a submerged 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV illumination. A PCR reaction mixture without DNA template was used as negative control for extraneous nucleic acid contamination.

2.4. Molecular Serotyping of *L. monocytogenes*

Listeria monocytogenes were separated into four major serotypes (1/2a, 1/2c, 1/2b and 4b) by multiplex-PCR described by Doumith et al. [25] using the five primers sets lmo0737, lmo1118, ORF2819, ORF2110 and prs. Four groups of serotypes are observed based on PCR results. Serogroups 1/2a-3a and 1/2b-3b-7 displays single fragments, 691 bp and 471 bp, with the primers lmo0737 and ORF2819 respectively. Serogroup 1/2c-3c displays two fragments (691 bp and 906 bp) when amplified with the primer sets lmo0737 and lmo1118, respectively. Similarly, serogroup 4b-4d-4e displays two fragments of size 471 bp and 597 bp when amplified with the primers ORF2819 and ORF2120 respectively. All members of the genus *Listeria* show single fragment of 370 bp when amplified with prs primers.

2.5. Detection of Virulence Associated Genes in *L. monocytogenes*

Detection of the virulence-associated genes in *L. monocytogenes* was carried out in two separate multiplex PCR assays. Internalin genes (*inlA*, *inlB*, *inlC*, and *inlJ*), were detected in a multiplex-PCR using primers and cycling conditions described by Liu et al. [26]. For the detection of *plcA*, *actA*, *hlyA*, *iap* and *prfA*, PCR assay was carried out as described by Kalorey et al. [27].

2.6. Determination Antimicrobial Resistance among *L. monocytogenes* Isolates

Sixty-two identified *L. monocytogenes* isolates were tested for their susceptibility to antibiotics using the broth micro-dilution method recommended by the standard criteria of the Clinical and

Laboratory Standards Institute (CLSI) guidelines M45-A2 for *L. monocytogenes* [28]. The antibiotics used include Amoxicillin, Ampicillin, Cefepime, Chloramphenicol, Ciprofloxacin, Clindamycin, Doxycycline, Erythromycin, Gentamycin, Kanamycin, Neomycin, Penicillin, Rifampin, Tetracycline, and Vancomycin. MIC was determined by two-fold dilution of antibiotics (in the range of 64 to 0.06 µg/mL) in cation-adjusted Mueller–Hinton broth (CAMHB, Difco Laboratories) supplemented with 5% lysed horse blood. The MIC breakpoints used for the interpretation of susceptibility were based on those proposed by Bertsch et al. [16] and Acar et al. [29].

3. Results and Discussion

3.1. Prevalence of *L. monocytogenes* in Raw Milk, Boiled Milk and Nunu

In this study, a total of 254 samples of raw milk, boiled milk and nunu were examined for *Listeria* spp. and *Listeria monocytogenes* (Table 1). Overall, 44/254 (12.2%) samples were contaminated with *Listeria* spp. whereas 14/254 (5.5%) samples were positive for *Listeria monocytogenes*. The prevalence of *L. monocytogenes* among the various samples were 8.8% (10/114) for raw cow milk and 4.7% (4/84) for nunu. No *Listeria* spp. or *L. monocytogenes* was detected in boiled milk.

Table 1. Prevalence of *Listeria* spp. and *Listeria monocytogenes* in raw milk, boiled milk and nunu.

Product	pH of Samples	Number of Samples	<i>Listeria</i> spp. (%)	<i>Listeria monocytogenes</i> (%)
Raw cow milk	6.7 ± 0.2	114	20 (17.5)	10 (8.8)
Boiled milk	6.3 ± 0.4	56	ND	ND
Nunu ^a	4.1 ± 0.9	84	11 (13.1)	4 (4.7)
Total	NA	254	44 (12.2)	14 (5.5)

ND: Not detected; NA: Not applicable; ^a Spontaneously fermented yoghurt-like milk beverage.

All *Listeria* spp. and *L. monocytogenes* isolates from raw milk and nunu samples were confirmed by both biochemical tests and duplex PCR (Figure 1).

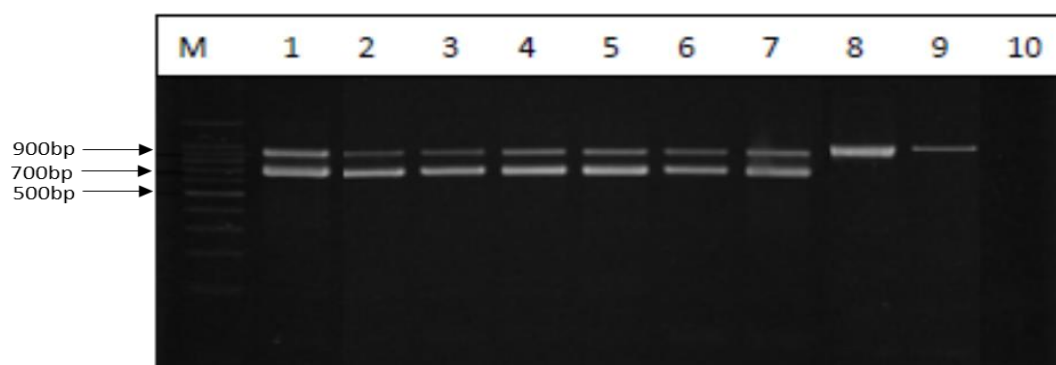


Figure 1. Representative PCR product detecting the highly conserved 16Sr RNA gene sequence for *Listeria* spp. and listeriolysin O gene sequence for *L. monocytogenes*. Lane M, 100 bp molecular size DNA marker; lane 1, *L. monocytogenes* ATCC 19115 (positive control); lanes 2–7, *L. monocytogenes* isolated from raw cow milk and nunu; lanes 8 and 9, non-*L. monocytogenes* isolated from raw cow milk and nunu; lane 10, negative control (PCR mixture without DNA template).

Among other human pathogenic microorganisms, *L. monocytogenes* is considered a major microbiological hazard associated with the consumption of raw cow milk [30,31]. The prevalence of *Listeria* spp. and *Listeria monocytogenes* in milk and dairy products, has been widely reported [17,31–33]. The prevalence of *L. monocytogenes* in milk varies considerably among different survey reports and has

been attributed to factors such as geographic (environmental) conditions, farm size, farm management practices, sampling and detection methods used among others [30]. In this report, the prevalence of *L. monocytogenes* in raw cow milk collected from small dairy farms in northern Ghana was 8.8% while Appiah et al. [20] reported prevalence of over 42% for raw cow milk sampled from farms in Accra (southern Ghana). According to other previous survey reports, the frequency of detection of *L. monocytogenes* in raw cow milk can vary from 0% [34] to more than 45% [35–40]. Despite the widely reported variation in the prevalence of *L. monocytogenes* in milk, all the surveys demonstrate that raw cow milk and dairy products can be a source of *Listeria* infections in humans [30,32].

Listeria monocytogenes occurs widely in agricultural and food processing environments [41] and can contaminate raw milk during milking processes [31]. Additionally, *L. monocytogenes* is widely distributed in food processing environments with the capacity to tolerate and grow in the temperature range of 0–45 °C in media with pH range of 4.4–9.4 [42–44].

Due to the observed high prevalence of *L. monocytogenes* in raw cow milk from dairy farms in this study, it is possible that the pathogen could enter the processing environments and persist during processing, resulting in the contamination of the dairy products. Nunu is produced by spontaneously fermenting raw cow milk at ambient temperature (28–35 °C) for about 18–24 h under uncontrolled conditions, bringing the pH to 4.1 ± 0.9 (Table 1). The fermented yoghurt-like milk beverage, nunu, can then be consumed within 4 days after fermentation without refrigeration or refrigerated at 4 °C for several weeks [21]. The observed prevalence of *L. monocytogenes* in nunu may be attributed to factors such as post-processing contamination, improper handling of raw milk, or inadequate fermentation to bring the pH below tolerance levels for *L. monocytogenes*. Thus, the production of organic acids and bacteriocins which are important natural antimicrobials in nunu, resulting from lactic acid fermentation of milk, might not be sufficient to eliminate *L. monocytogenes* in the product and therefore should not replace good hygiene practices (practices) during processing. On the other hand, *Listeria* was not detected in boiled milk, which undergoes thermal treatment without further processing. Boiling is one method that is known to reduce or even eliminate the microbiological risk associated with the consumption of raw milk [30].

3.2. Molecular Serogroups of *L. monocytogenes* Isolates

Based on PCR serotyping, *L. monocytogenes* isolates belonged to molecular serogroups 1/2a-3a (32/62, 51.6%), 1/2b-3b-7 (14/62, 22.6%), 1/2c-3c (7/62, 11.3%) and 4b-4d-4e (9/62, 14.5%). Previous reports indicate that over 95% of the isolates originating from human listeriosis and food samples belonged to serotypes 1/2a, 1/2b, 1/2c, and 4b [6,11,45–47]. Consistent with the previous reports, 51.6% of *L. monocytogenes* isolates in this study belonged to serogroup 1/2a-3a, followed by 1/2b-3b-7 (22.6%), 4b-4d-4e (14.5%) and the 1/2c-3c (11.3%). The high prevalence and enhanced ability of serotype 1/2a to persist in food environments has been ascribed to the fact that members of this group might be carrying more plasmids which often confer resistance to toxic compounds [48]. Major outbreaks of invasive forms of listeriosis have been associated with serotype 4b [49], which has also been isolated from animal-derived foods [6]. Thus, the frequent detection of serotype 4b in human listeriosis outbreaks and sporadic cases indicate that these strains may be more virulent than other serotypes.

3.3. Prevalence of Virulence-Associated Markers

Irrespective of serotype, all of the sixty-two (62) *L. monocytogenes* tested harbored the virulence-associated genes *inlA*, *inlB*, *inlC*, and *inlJ*. *inlA*, *inlB*, *inlC*, *inlJ*, *plcA*, *actA*, *hlyA*, *iap* and *prfA*. Listeriolysin O is reported to be the main bacterial determinant for the escape of *L. monocytogenes* from both primary and secondary vacuoles and is one of its main virulent factors [50,51]. The detection of internalin genes (*inlA*, *inlB*, *inlC*, and *inlJ*) in almost all examined *L. monocytogenes* isolates from food samples have been reported [52–55]. The virulence markers *InlA*, *InlC* and *InlJ*, all of which play various roles in *L. monocytogenes* infections and pathogenesis, are important virulence factors of *L.*

monocytogenes [11]. In *L. monocytogenes*, InlA and InlB have evidently been shown to be important for host cell invasion and virulence [56,57]. Furthermore, null mutations in the internalin genes (*inlA*, *inlB*, *inlC*, and *inlJ*) result in reduced invasion or virulence in tissue culture or animal model studies [58–60], underscoring the important role of these genes in virulence and pathogenesis. In general, the presence of the internalin genes and the other virulence-associated genes in *L. monocytogenes* isolated from milk and dairy products in Ghana indicates that these *L. monocytogenes* strain could be potentially virulent.

3.4. Antibiotic Resistance among *L. monocytogenes* Isolates

Phenotypic resistance to 15 antimicrobial agents by 62 *L. monocytogenes* isolates from raw milk and nunu in Ghana are shown in Table 2. Based on the MIC breakpoints used in this study, *L. monocytogenes* isolates were generally susceptible to amoxicillin (100%), ampicillin (100%), erythromycin (100%), gentamycin (100%), penicillin (100%), rifampicin (100%) and vancomycin (100%). However, phenotypic resistances were observed against neomycin (61.3%) and tetracycline (24.2%), while intermediate susceptibilities were obtained for chloramphenicol, ciprofloxacin, clindamycin, doxycycline, kanamycin, neomycin, streptomycin and tetracycline.

Table 2. Antimicrobial susceptibility profile of *L. monocytogenes* isolates.

Antimicrobial	^a MIC Breakpoints		^b Interpretations n (%)		
	S (≤)	R (≥)	S	I	R
Amoxicillin	2	8	62 (100)	0	0
Ampicillin	4	8	62 (100)	0	0
Chloramphenicol	8	32	60 (96.8)	2 (3.2)	0
Ciprofloxacin	2	8	55 (88.7)	7 (11.3)	0
Clindamycin	2	8	51 (82.3)	11 (17.7)	0
Doxycycline	4	16	58 (93.5)	4 (6.5)	0
Erythromycin	2	4	62 (100)	N/A	0
Gentamycin	2	8	62 (100)	0	0
Kanamycin	16	64	57 (91.9)	5 (8.1)	0
Neomycin	8	32	21 (33.9)	3 (4.8)	38 (61.3)
Penicillin	4	8	62 (100)	0	0
Rifampicin	1	2	62 (100)	N/A	0
Streptomycin	8	32	61 (98.4)	1 (1.6)	0
Tetracycline	2	8	44 (70.9)	3 (4.8)	15 (24.2)
Vancomycin	8	32	62 (100)	0	0

^a MIC breakpoint values for all antibiotics against *L. monocytogenes* were according to the description of Bertsch et al., 2014, except doxycycline and neomycin for which the breakpoints proposed by Acar et al., 1998 were used. ^b S: susceptible; I: intermediate susceptibility; R: resistant.

Listeria monocytogenes is naturally reported to be sensitive to a wide range of antibiotics. However, recent reports show a continuous pattern of emergence of resistant strains [6,14,48]. *Listeria monocytogenes* from food sources have generally been reported to be highly susceptible to ampicillin, gentamycin, penicillin, rifampicin and vancomycin. On the other hand, varying prevalence of resistance to tetracycline by *L. monocytogenes* isolates from different food sources have also been reported [16,53,61,62]. The high number of *L. monocytogenes* isolates showing intermediate susceptibility to ciprofloxacin (11.3%) and clindamycin (17.7%) has similarly been reported by Lyon et al. [63] and Bertsch et al. [16]. Natural populations of several *Listeria* species have been described as intermediately susceptible to ciprofloxacin and clindamycin [64].

4. Conclusions

The results presented in this report demonstrates *Listeria monocytogenes* is prevalent in raw milk and nunu, a spontaneously fermented yoghurt-like milk beverage, in Ghana. *L. monocytogenes* isolates from raw milk and nunu in Ghana harbored virulence-associated genes. However, the *L. monocytogenes*

isolates were generally susceptible to the different antibiotics tested except tetracycline and neomycin for which phenotypic resistance was observed. However, genotypic assessment of the prevalence of genetic markers associated with antimicrobial resistance for the genetic basis of resistance phenotypes observed in this study would provide a better baseline for further antimicrobial resistance molecular surveillance of *L. monocytogenes* milk and dairy products in Ghana. Implementation of adequate sanitation program and good hygiene/manufacturing practices (GHP/GMP) for milk production and processing of fermented dairy beverages in Ghana could reduce microbial contamination of these products.

Author Contributions: J.O.-K. conceived the research concept, participated in laboratory experiments, analyzed and interpreted the data and wrote the manuscript. A.W. carried out most of the laboratory experiments and participated in analysis and interpretation of data. F.A. participated in interpretation of data and reviewed the manuscript. L.J. supervised the project and corrected the manuscript. All authors read and approved the content of the manuscript.

Acknowledgments: The work was supported with funding from Danida (Danish International Development Agency, Ministry of Foreign Affairs) and the Government of Ghana (GoG) through the collaborative research project ‘Preserving African Food Microorganisms for Green Growth’ DFC No. 13-04KU. The authors are also grateful to management of the Savana Agricultural Research Institute (SARI), Tamale-Ghana and Navrongo Health Research Centre (NHRC), Ghana, for granting access to their laboratory facilities.

Conflicts of Interest: The authors declare no conflict of interest in this work.

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